doi: 10.17113/ftb.55.01.17.4770

Assessment of Grape, Plum and Orange Synthetic Food Flavourings Using *in vivo* Acute Toxicity Tests

Ila Monize Sousa Sales[1], *Janaína Sousa Barbosa*[1], *Fabelina Karollyne Silva dos Santos*[1], *Felipe Cavalcanti Carneiro da Silva*[1,2], *Paulo Michel Pinheiro Ferreira*[2], *João Marcelo de Castro e Sousa*[1,2] *and Ana Paula Peron*[1,3]*

[1]Laboratory of Cytogenetics and Mutagenesis (LaCM), Biological Sciences Academic Course, Campus Senador Helvídio Nunes de Barros (CSHNB), Federal University of Piauí (UFPI), Picos, 64049-550 Piauí, Brazil

[2]Department of Biophysics and Physiology, Postgraduate Programs in Pharmaceutical Sciences, Center of Health Sciences, Federal University of Piauí (UFPI), Teresina, 64049-550 Piauí, Brazil

[3]Postgraduate Program in Genetics and Improvement, Federal University of Piauí (UFPI), Teresina, 64049-550 Piauí, Brazil

Received: May 2, 2016
Accepted: October 5, 2016

Summary

The present study evaluates the acute toxicity of synthetic grape, plum and orange flavourings in root meristem cells of *Allium cepa* at the doses of 3.5, 7.0 and 14.0 mL/kg and exposure times of 24 and 48 h, and in bone marrow erythrocytes of mice treated orally for seven days with 0.5, 1.0, 2.0, 5.0 and 10.0 mL/kg of flavouring. The results of the plant test showed that grape, plum and orange flavourings, at both exposure times, inhibited cell division and promoted the formation of a significant number of micronuclei and mitotic spindle changes. These alterations were observed in at least one exposure time analysed, demonstrating a significant cytotoxic, genotoxic and mutagenic activity. In mouse bioassay, animals treated with 2.0, 5.0 and 10.0 mL/kg of flavouring died before the seventh day of treatment. The amounts of 0.5 and 1.0 mL/kg of the three additives were cytotoxic to erythrocytes, and treatment with the grape flavouring significantly induced the formation of micronucleated cells in the bone marrow of animals. Therefore, under the study conditions, the grape, plum and orange flavouring additives promoted significant toxicity to cells of the test systems used.

Key words: aroma and flavour additives, toxicity, cell division, mitotic spindle changes, micronucleus

Introduction

Globalisation and the development of new technologies have caused significant changes in the human eating habits, who, in recent decades, have frequently consumed foods rich in chemical additives (*1*). These substances, also called microingredients, include synthetic flavourings of special importance for the food industry as they provide sensory properties of aroma and flavour to all kinds of processed foods (*2*,*3*). These synthetic additives have a complex formulation containing diverse chemical compounds, such as diluents, preservatives and colourants, among others (*4*,*5*) and are classified by the food industry into nature-identical and artificial additives (*2*).

Worldwide, flavour and aroma additives are standardised and released for use by food safety agencies Food and Agriculture Organization (FAO) and Flavour and Extract Manufacturer Association (FEMA) (*2*,*3*), and nation-

*Corresponding author: Phone: +55 89 3422 4200, E-mail: anapaulaperon@ufpi.edu.br

ally by the National Sanitary Surveillance Agency (ANVISA) through RDC Resolution 2 of January 15, 2007 (*4*). However, these regulatory agencies do not report in detail which compounds and concentrations are found in these microingredients, and to date also have not defined the acceptable daily intake (ADI) and the tolerable dose limit of flavouring for each food type (*1,2,4,6,7*). Moreover, the enforcement agencies do not require industrialised food manufacturers to display on product labels the description of the substances that make up the flavouring used in each product (*4*). Similarly, these agencies do not require the additive manufacturers to give the description on the label of the chemical components found in the aroma and flavour additives (*5–7*).

Because of this lack of information, Konishi *et al*. (*8*), Koca *et al*. (*5*), Marques *et al*. (*3*) and Moura *et al*. (*1*) highlight the priority for evaluation of the cytotoxic, genotoxic and mutagenic potential of microingredients of aroma and flavour. Similarly, the FEMA and ANVISA emphasise the need for studies on acute toxicological effect in different bioassays, with the justification for obtaining the data indicating the need for more detailed research on the toxicity of these substances (*2,4,9*).

Cytotoxic, genotoxic and/or mutagenic compounds can alter vital cellular mechanisms, such as gene replication and transcription, and promote mitotic spindle alterations and chromosomal breaks. These changes can significantly impair cell division of the affected tissue or organ, and trigger and/or potentiate cancerous processes (*10–12*). According to Zaineddin *et al*. (*13*), Moura *et al*. (*1*) and Santana *et al*. (*14*), the development of the most common types of cancer results from the interaction between endogenous and environmental factors, remarkably the diet, particularly when composed of processed foods in excess.

Root meristem cells of *Allium cepa* L. (onion) are an efficient test system for initial screening of acute cytotoxicity and genotoxicity of chemicals (*14,15*). This bioassay provides excellent proliferation kinetic properties and a few chromosomes (2*n*=16), which facilitates the detection of mitotic spindle changes and chromosomal breaks (*16,17*). It also allows the verification of changes in the cell division or mitotic index when exposed to chemical compounds with potential cytotoxic action (*3,16*), and shows mostly a satisfactory similarity to results obtained in other bioassays (*1,3*).

Among the methods assessing the damage caused by cytotoxic and/or mutagenic substances in animals, the micronucleus test carried out preferably in blood tissues of high proliferative activity stands out, since their cells are frequent targets of clastogenic agents (*18*). This test allows, by means of micronucleated erythrocyte frequency, to infer whether the tested substances or chemicals cause disorders, such as breaks and chromosome bridges and/or chromosomal delays during cell division (*18,19*), and also to evaluate the cytotoxicity of substances through reduction of erythropoiesis in the analysed tissue. In this way, this bioassay is considered the main *in vivo* evaluation of acute exposure among cytotoxicity tests and therefore a very important parameter in the evaluation of safety in the use of compounds or chemicals in general (*20*).

Thus, the present study analyses the cytotoxic, mutagenic and genotoxic potential of synthetic, nature-identical, grape, plum and orange flavourings with the use of root meristem cells of *A. cepa* and femoral bone marrow of mice. These additives were chosen for analysis because they are used extensively by the food industry in the manufacture of processed sweet foods, such as processed fruit juices, milk drinks, ice cream, jellies, candies, jams, mixed alcoholic drinks and liquors.

Material and Methods

Food flavourings

In this study, no dilution was made to set the doses of flavourings, that is, the toxicity of these additives was tested directly from the original solutions sold on the market. This is because the aroma additives have complex chemical formulation not described in detail by food safety agencies, and so, the concentration and the action of compounds present in these microingredients could be altered if diluted. It is also important to mention that the formulation of all synthetic food flavouring standards throughout the word and in Brazil is regulated by ANVISA (*4*).

Synthetic, nature-identical, grape, plum and orange liquid flavourings were obtained from a retailer specialised in national and international marketing of synthetic food additives (Trajano Food Addittives, Recife, Brazil). The label of the three flavorings suggested the use of 7.0 mL per kg of mass, so the first dose set for the study was 7.0 mL/kg and the other two were 3.5 and 14.0 mL/kg. Flavouring solutions were stored in 100-mL amber bottles and were used before expiry date. Toxicity tests on *A. cepa* root meristem were performed between September and November 2015.

As mentioned previously, to date, there is no acceptable daily intake (ADI) set for flavourings in general and there are no studies in the literature on the toxicological assessment of these substances in animal assays. Thus, for the assessment of the toxicity of grape, plum and orange additives, the doses of 0.5, 1.0, 2.0, 5.0 and 10.0 mL were determined based on the method proposed by Miller and Tainter (*21*).

Determination of cytotoxicity by Allium cepa assay

Onion bulbs were placed in aerated bottles with distilled water at room temperature (approx. 27 °C) until obtaining approx. 2.0 cm long roots. Five onion bulbs per each experimental group were used. Before treatments, some roots were fixed in Carnoy's solution 3:1 (ethanol/acetic acid) for 24 h and used as control.

Next, the remaining roots were placed in their respective solutions of flavouring for 24 h and then some roots were taken and fixed in Carnoy´s solution. Subsequently, the rest of the roots on each bulb were returned to their respective solutions, where they remained for another 24 h. Then, the roots were collected and fixed again. The exposure times of 24 and 48 h were set to evaluate the effects of flavourings on more than one cell cycle. At each collection, on average, three roots per bulb were taken.

On average, three slides were mounted per bulb, following the protocol proposed by Guerra and de Souza (*22*), and analysed under an optical microscope (Zeiss, São Paulo, Brazil) with 40× objective lens. Per each bulb, 1000 root cells were analysed, totalling 5000 cells of the control, and root cells treated for 24 and 48 h. Cells in interphase, prophase, metaphase, anaphase and telophase were examined.

The number of interphase and dividing cells was calculated for each control and exposure time and the cell division or mitotic indices for evaluation of the cytotoxic effect were determined. Mutagenic action of flavourings was analysed by determining the number of micronucleated cells, and genotoxicity by mitotic spindle changes.

Cytotoxic effect of synthetic flavourings on mice

In order to evaluate the toxicity of grape, plum and orange flavourings in an animal bioassay, Swiss mice (*Mus musculus* L.), males and females, three months of age and 50 g of average body mass, were provided by the Central Animal House, Federal University of Piauí (Teresina, Brazil). During the experiment, mice were kept in plastic cages at a constant temperature of (25±2) °C and 12 h light/dark cycle, and fed standard commercial chow and water *ad libitum*.

Mice used in this study were treated according to the principles set by the Brazilian College on Animal Experimentation (COBEA) (*23*) and in accordance with the requirements of Brazilian law (*24*). The experimental protocol with rodents developed in this study was previously approved by the Ethics Committee for Animal Experimentation (CEEA) of the Federal University of Piauí (opinion 008/2015).

A total of seven experimental groups were established for the analysis of flavourings: two control groups, one consisting of non-treated animals, and the other containing animals treated with the doses of 50 mg/kg of cyclophosphamide (Merck, São Paulo, Brazil), equivalent to 36.45 % lethal dose; and five groups of animals treated with grape, plum or orange flavourings (in mL/kg): 0.5, 1.0, 2.0, 5.0 and 10.0.

For each experimental group, three mice were randomly selected according to gender. Flavourings were administered *via* gavage in a single daily application for seven days using a syringe for oral administration of small dosages. Mice of the positive control group received a different treatment consisting of cyclophosphamide at 50 mg/kg intraperitoneally, only 24 h before the sacrifice. On the eighth day, after sacrifice by cervical dislocation, femora were removed by surgery for bone marrow extraction.

Bone marrow extracted from mice was inserted into medium for karyotyping and then centrifuged in a tube twice at 1000×*g* (model MIKRO 185 centrifuge; Hettich Lab Technology, Beverly, MA, USA) for 5 min. A drop of the suspension obtained from each animal was spread on a slide. After drying, the material on the slides was fixed in pure methanol for 10 min, allowed to dry at room temperature with Giemsa (Merck) diluted in phosphate buffer, pH=6.8, at a ratio of 1:10 for 15 min. After staining, the slides were washed in distilled water and air-dried.

The bone marrow material was analysed under oil immersion microscope (Zeiss Microscopy Brasil, São Paulo, Brazil) in which for each smear, 200 polychromatic erythrocytes (PCE) were examined. To determine the cytotoxicity, a total of 400 PCE and normochromatic erythrocytes (NCE) were counted per animal (200 per blade) and the PCE frequency was determined as a ratio of PCE and PCE+NCE. For mutagenic assessment, micronucleated cells were counted in 1000 erythrocytes per animal.

Statistical data analysis

The results obtained in *Allium cepa* were analysed by χ^2 statistical analysis ($p<0.05$). Data obtained from the animal bioassay were tested by ANOVA followed by Tukey's *post-hoc* test using STATISTICA v. 7.0 software (Dell, São Paulo, Brazil), and $p<0.05$ was adopted as significance level.

Results and Discussion

Based on the results for grape flavouring in Table 1, it is observed that after the exposure of *Allium cepa* root meristem cells to the dose of 3.5 mL/kg for 24 h, the mitotic index was not significantly different from its control. However, the cell division index after the exposure for 48 h to the same dose was statistically lower than the respective control and the above result. At doses of 7.0 and 14.0 mL/kg of this same flavouring, cell division index after the exposure for 24 and 48 h was significantly lower than those of their controls. In addition, comparison of the values of mitotic index between the samples exposed for 24 and 48 h did not show significant differences.

After the exposure of *A. cepa* cells to 3.5 mL/kg of the plum flavouring for 24 and 48 h, a significant decrease in cell division was observed compared to the mitotic index of its respective control (Table 1). The cell division index of samples exposed for 24 h was significantly different from the value after 48-hour exposure, when cell proliferation decreased dramatically with the increase of the exposure time. In turn, the values of cell proliferation after the exposure to 7.0 and 14.0 mL/kg of plum flavouring and 3.5, 7.0 and 14.0 mL/kg of the orange flavouring, as seen in Table 1, after both exposure times were significantly lower than those registered of their respective controls. However, there were significant differences in mitotic index values between the samples exposed for 24 and 48 h to the same amounts of plum and orange microingredients.

According to the results in Table 1, all tested amounts of grape, orange and plum flavouring were cytotoxic to root meristem cells of *A. cepa* at both exposure times, which is confirmed by significant antiproliferative effect caused to the cells.

Table 2 shows that 3.5 mL/kg of the grape flavouring did not cause significant damage to the meristem cells after 24 h of exposure. However, this dose after 48 h of exposure caused significant alterations to the cells compared to its respective control and the results after 24 h of exposure. Nevertheless, the doses of 7.0 and 14.0 mL/kg of grape flavouring induced the development of a significant number of cellular abnormalities after 24 h of exposure. All doses of the plum flavouring induced a signifi-

Table 1. Number of cells observed in each phase of the cell cycle in root meristem tissue of *Allium cepa* treated with water and synthetic grape, plum and orange flavourings

(*V*/*m*)/(mL/kg)	*t*(exposure)/h	*N*						MI/%
		TCII	P	M	A	T	TCD	
Grape flavouring								
	control	3320	894	297	294	195	1680	33.6[a]
3.5	24	3673	743	198	195	191	1327	26.5[a]
	48	4442	162	129	121	146	558	11.2[b]
	control	3469	767	233	260	271	1531	30.6[a]
7.0	24	4445	178	149	118	110	555	11.1[b]
	48	4539	126	113	114	108	461	9.2[b]
	control	3605	753	322	167	153	1395	27.9[a]
14.0	24	4364	265	91	92	98	546	10.9[b]
	48	4520	238	87	79	76	480	9.6[b]
Plum flavouring								
	control	3992	518	135	183	172	1008	20.2[a]
3.5	24	4486	181	126	161	46	514	10.3[b]
	48	4832	79	39	38	12	168	3.4[c]
	control	4040	447	193	178	142	960	19.2[a]
7.0	24	4668	143	113	47	29	332	6.7[b]
	48	4775	81	67	33	44	225	4.5[b]
	control	4027	529	200	152	92	973	19.5[a]
14.0	24	4559	178	97	34	32	341	6.8[b]
	48	4731	143	73	24	29	269	5.4[b]
Orange flavouring								
	control	4255	241	194	162	148	745	14.9[a]
3.5	24	4859	60	26	34	21	141	2.8[b]
	48	4849	55	33	47	151	151	3.2[b]
	control	4324	207	143	192	134	676	13.5[a]
7.0	24	4778	94	34	49	45	222	4.4[b]
	48	4897	47	20	24	12	103	2.1[b]
	control	4532	219	121	113	115	568	11.4[a]
14.0	24	4789	63	61	38	49	211	4.2[b]
	48	4871	57	23	21	28	129	2.6[b]

V=volume of the flavouring solution, *m*=mass (on average 0.2 kg) of onion bulb used, TCII=total number of interphase and undifferentiated cells, P=prophase, M=metaphase, A=anaphase, T=telophase, TCD=total number of dividing cells, MI=mitotic index. Within the same treatment, MI values followed by different letters are significantly different at 5 % by χ^2 test

cant number of mitotic spindle changes and micronuclei in root cells 24 and 48 h after the exposure. All three doses of orange flavouring did not induce a significant number of alterations in the examined cells after both exposure times. Therefore, it can be concluded that the amount of 3.5 mL/kg of grape flavouring after 48 h of exposure and 3.5, 7.0 and 14.0 mL/kg of plum flavouring, after both exposure times, proved to be genotoxic and mutagenic to the tested *A. cepa* cells.

The significant number of micronuclei and chromosomal alterations observed in root meristems treated with grape and plum flavourings (Table 2) confirms the antiproliferative effect in root meristem cells (Table 1). According to Santana *et al*. (*14*), inhibition of cell division is related to cell death caused by disturbances, such as toxic action of chemical substances or compounds to cell division kinetics or essential chromosomes or cells. These events, according to Gomes *et al*. (*25*) and Marques *et al*. (*3*), cause significant reduction in cell replacement and alter protein synthesis of the tissue or organ where they occur.

Regarding the evaluation of flavouring toxicity to mice, animals treated with 2.0, 5.0 and 10.0 mL/kg of grape, plum or orange flavouring died on days three, four and five of the experiment, respectively. From the third day of treatment, all three animals treated with 10 mL/kg of grape microingredient had severe abdominal swelling. Unfortunately, it was not possible to determine the LD_{50} (median lethal dose) of the three additives with the doses evaluated herein.

Table 2. Number and types of cellular abnormalities observed in root meristem cells of *Allium cepa* treated with water and synthetic grape and plum flavourings

(*V*/*m*)/(mL/kg)	*t*(exposure)/h	Colchicine metaphase	Anaphase bridge	Telophase bridge	Micronucleus	Binucleate cell	*N*(TCA)
Grape flavouring							
	control	0	0	1	0	0	1[a]
3.5	24	1	0	0	0	0	1[a]
	48	22	49	13	89	0	173[b]
	control	0	0	1	0	0	1[a]
7.0	24	9	72	22	111	3	217[b]
	48	0	37	59	98	1	195[b]
	control	0	1	0	0	0	1[a]
14.0	24	0	28	33	171	2	234[b]
	48	18	20	49	108	0	197[b]
Plum flavouring							
	control	1	0	0	0	0	1[a]
3.5	24	7	11	8	47	9	73[b]
	48	4	13	9	35	0	61[c]
	control	0	1	0	0	0	1[a]
7.0	24	9	13	13	59	0	94[b]
	48	9	17	9	62	0	97[b]
	control	1	0	0	0	0	1[a]
14.0	24	13	18	7	55	0	93[b]
	48	5	27	17	39	1	88[b]

V=volume of the flavouring solution, *m*=mass (on average 0.2 kg) of onion bulb used, *N*(TCA)=number of total cellular alterations. Within the same treatment, TCA values followed by different letters are significantly different 5 % by χ^2 test

The treatments with 0.5 and 1.0 mL/kg of the three flavourings (Table 3) altered the maturation cycle of mouse bone marrow cells, reducing the number of polychromatic erythrocytes, and thus, under the study conditions, these doses can be characterised as cytotoxic. These results corroborate those observed for *A. cepa* (Table 1), where the investigated doses of grape, plum and orange additives drastically reduced the cell division index of root meristems, indicating a strong cytotoxic activity.

Table 3 also shows that the doses of 0.5 and 1.0 mL/kg of grape flavouring had mutagenic potential because they induced a statistically significant formation of micronucleated erythrocytes. The results observed in *A. cepa* for this microingredient (Table 2) corroborate the data observed for grape flavouring in animal cells, where the treatments promoted cellular alterations, such as micronuclei, at a significant frequency. It is known that micronuclei, being acentric fragments that were not incorporated into the nuclei of cells during telophase, can cause cell death due to suppression or reduction of expression of primary or primordial genes. Thus, the significant presence of micronuclei in tissue promotes systemic cytotoxicity, resulting from high mutation rates and, consequently, genetic instability in cells (*26*). Importantly, although genetic toxicity is not a measure of carcinogenicity, it is often related to the onset of cancer, since there is a positive correlation between the increased frequency of micronuclei and the appearance of tumours in mammals (*27*).

Table 3. Cytotoxic and mutagenic potential of grape, plum and orange flavourings determined in femoral bone marrow cells of mice (*Mus musculus*) treated orally for seven days

Group	(*V*/*m*)/(mL/kg)	Cytotoxic activity	MNF
Negative control	No treatment	1.12±0.32	3±2.5
Positive control	Cyclophosphamide	(0.5±0.11)[a]	(15.3±4.2)[a]
Grape flavouring	0.5	(0.6±0.09)[a]	(10.66±9.85)[a]
	1.0	(0.5±0.12)[a]	(23.0±4.4)[a]
Plum flavouring	0.5	(0.7±0.14)[a]	3.33±2.06
	1.0	(0.7±0.07)[a]	3.16±0.75
Orange flavouring	0.5	(0.25±0.2)[a]	1.8±1.5
	1.0	(0.2±0.1)[a]	5.7±3.2

V=volume of the flavouring solution, *m*=mass of the used animal, MNF=micronucleus frequency. ANOVA followed by Tukey´s test: [a]significant compared to the negative control at $p<0.05$

Unfortunately, the chemical composition of grape, plum and orange flavourings was not found in the literature or on the labels. However, the scientific literature demonstrates the toxicity at the cellular level of chemical constituents with diluent and preservative activities, according to RDC Resolution (*4*) on the basic formulation of flavourings, and corroborates the data obtained for the three flavourings evaluated in this study. Among these

compounds benzoic alcohol stands out, as it is responsible for maintaining uniformity and facilitating incorporation and dispersion of the flavour in food products. Analysing the action at the cellular level of this diluent, Demir *et al*. (*28*) found that the alcohol promoted significant damage to the mitotic spindle and therefore to cell division in human peripheral blood cells.

Furthermore, diacetyl (butane-2,3-dione) is another diluent found in the formulation of flavourings. In lymphoma gene mutation assay in rats, Whittaker *et al*. (*29*) reported that this compound caused significant damage to the loci on chromosome 11 of these cells, causing loss of the enzyme thymidine kinase gene expression. Likewise, More *et al*. (*7*) observed that the diluent diacetyl had the potential to replace thymine with guanine in euchromatin regions and caused the disruption of hydrogen and disulfide bonds in the tertiary structure of enzymes involved in the cell division.

In turn, among the chemical constituents responsible for delaying the action of microorganisms, enzymes and physical agents in flavouring solutions, potassium benzoate, sodium benzoate and potassium nitrate (*9*) are preservatives that, according to Mpountoukas *et al*. (*30*) and Zequin *et al*. (*6*), were cytotoxic and genotoxic to normal human peripheral blood cells. The flavourings also contain the preservatives boric acid, citric acid, potassium citrate and sodium citrate (*9*), which, in agreement with Türkoğlu (*31*), resulted in significant reduction of the cell division index of root meristem cells of *A. cepa*, proving to be cytotoxic.

Currently, the only class of compounds in the formulation of food flavourings that have restricted use standardised by food safety agencies is the class of extraction solvents, among which the agaric acid, aloin, β-asarone, berberine, coumarin, hydrocyanic acid, hypericin, pulegone, quassin, safrole and isosafrole, santonin and α- and β-thuyone have maximum tolerable limits legally determined (*4,9*). Nevertheless, according to Moura *et al*. (*1*) and Konishi *et al*. (*8*), the composition of flavourings in general includes 11 classes of chemical compounds, where each consists of, on average, 20 chemical compounds, which have not been evaluated for their cytotoxic, mutagenic and genotoxic potential.

In relation to the toxicity of flavourings in general, ANVISA (*4*), although not mentioning which studies, concentrations and compounds, or which flavourings have led to such a conclusion, stated that high doses of such synthetic additives cause annoying and narcotic actions in the organism and may cause toxicity in digestive tract when used chronically and indiscriminately (*4,9*). Further, Salinas (*32*) and Polônio and Peres (*33*) state that the use of flavourings at low doses does not pose risk to human health; however, when used in doses higher than recommended, these substances can cause annoying and narcotic actions besides chronic cellular toxicity in the long term. Also, these authors did not specify which doses or concentrations of these additives are considered to be high or low, and they do not discriminate which flavourings or test organisms were used to obtain this information.

In this way, although the use of flavourings is permitted by EFSA, FEMA (*10,25*) and ANVISA (*4*), there is a pressing need for more detailed medium- and long-term studies using different tests, dosages and time of exposure to determine the toxicity of these substances. Furthermore, our findings, although preliminary, together with the results of the evaluation of toxicity at the cellular level of compounds in the formulation of flavourings already conducted, indicate the need to define the chemical composition of flavourings in general using high performance liquid chromatography to properly determine the toxicity of these additives and ensure the safety of consumers.

Conclusion

Synthetic grape, plum and orange flavourings under the study conditions caused acute toxicity to root meristem cells of *Allium cepa* and to femoral bone marrow cells of mice. The results of toxic activity obtained at the cellular level of grape, plum and orange food flavourings are of utmost importance because, to date, there are no toxicity studies on these additives and no daily intake level has been set for such microingredients. Also, these flavouring solutions are present in many processed foods and are freely traded in retail markets and websites specialised in the sale of food additives.

References

1. Moura AG, Santana GM, Ferreira PMP, Sousa JMC, Peron AP. Cytotoxicity of cheese and Cheddar cheese food flavorings on Allium cepa L root meristems. Braz J Biol. 2016;76: 439–43. http://dx.doi.org/10.1590/1519-6984.20514
2. Xu Z, Gu C, Wang K, Ju J, Wang H, Ruan K, Feng Y. Arctigenic acid, the key substance responsible for the hypoglycemic activity of Fructus arctii. Phytomedicine. 2015;22:128–37. http://dx.doi.org/10.1016/j.phymed.2014.11.006
3. Marques GS, Silva SIO, Sousa JMC, Ferreira PMP, Peron AP. Cytotoxicity and mutagenic potential of liquid synthetic food flavoring evaluated individually and in association. Food Sci Technol. 2015;35:183–8. http://dx.doi.org/10.1590/1678-457x.6596
4. Good practices in the use of food additives. Resolution No. 05. Poços de Caldas, MG, Brazil: National Health Surveillance Agency, Ministry of Health; 2007. Available from: http://www.anvisa.gov.br/legis/resol/2007/rdc/02_170107rdc.pdf (in Portuguese).
5. Koca N, Erbay Z, Kaymak-Ertekin F. Effects of spray-drying conditions on the chemical, physical and sensory properties of cheese powder. J Dairy Sci. 2015;98:2934–43. http://dx.doi.org/10.3168/jds.2014-9111
6. Zequin N, Yüzbaşioğlu D, Ünal F, Ylmaz S, Aksoy H. The evaluation of the genotoxicity of two food preservatives: sodium benzoate and potassium benzoate. Food Chem Toxicol. 2011;49:763–9. http://dx.doi.org/10.1016/j.fct.2010.11.040
7. More SS, Raza A, Vince R. The butter flavorant, diacetyl, forms a covalent adduct with 2-deoxyguanosine, uncoils DNA, and leads to cell death. J Agric Food Chem. 2012;60: 3311–7. http://dx.doi.org/10.1021/jf300180e
8. Konishi Y, Hayashi SM, Fukushima S. Regulatory forum opinion piece: supporting the need for international harmonization of safety assessments for food flavoring substances. Toxicol Pathol. 2014;42:949–53. http://dx.doi.org/10.1177/0192623313495603

9. Technical regulation on flavoring additives/aroma. Resolution No. 104. Poços de Caldas, MG, Brazil: National Health Surveillance Agency, Ministry of Health; 1999. Available from: http://www.engetecno.com.br/port/legislacao/norqual_aditivos_aromatizantes.htm (in Portuguese).
10. de Oliveira MVA, Alves DDL, Lima LHGM, Sousa JMC, Peron AP. Cytotoxicity of erytrosine (E-127), brilliant blue (E--133) and red 40 (E-129) food dyes plant test system. Acta Sci Biol Sci. 2013;35:557–62. http://dx.doi.org/10.4025/actascibiolsci.v35i4.18419
11. Valavanidis A, Vlachogianni T, Fiotakis K, Loridar S. Pulmonary oxidative stress, inflammation and cancer: respirable particular matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms. Int J Environ Res Public Health. 2013;9:3886–907. http://dx.doi.org/10.3390/ijerph10093886
12. Zilifdar F, Alpen-Hayta S, Yilmaz S, Kaplan-Özen Ç, Foto E, Aydoğan N, et al. Genotoxic potential and eukaryotic DNA topoisomerase I inhibitory effects of some benzoxazine derivatives. Med Chem Res. 2014;23:480–6. http://dx.doi.org/10.1007/s00044-013-0658-5
13. Zaineddin AK, Buck K, Vrieling A, Heinz J, Flesch-Janys D, Linseisen J, Chang-Claude J. The association between dietary lignans, phytoestrogen-rich foods, and fiber intake and postmenopausal breast cancer risk: a German case-control study. Nutr Cancer. 2012;64:652–65. http://dx.doi.org/10.1080/01635581.2012.683227
14. Santana GM, Deus MSM, Sousa JMC, Ferreira PMP, Fernandes HB, Peron AP. Antimitotic and antimutagenic action of the Hymenaea stigonocarpa bark on dividing cells. Braz J Biol. 2016;76:520–5. http://dx.doi.org/10.1590/1519-6984.23014
15. Neves ESB, Ferreira PMP, Lima LHGM, Peron AP. Action of aqueous extracts of Phyllanthus niruri L. (Euphorbiaceae) leaves on meristematic root cells of Allium cepa L. An Acad Bras Cienc. 2014;86:1131–7. http://dx.doi.org/10.1590/0001-3765201420130170
16. Herrero O, Pérez Martín JM, Fernández Freire P, Carvajal López L, Peropadre A, Hazen MJ. Toxicological evaluation of three contaminant of emerging concern by use of the Allium cepa test. Mutat Res-Gen Tox En. 2012;743:2–4. http://dx.doi.org/10.1016/j.mrgentox.2011.12.028
17. Lacerda LP, Malaquias G, Peron AP. Antiproliferative action of aqueous extracts of Hymenaea stigonocarpa Mart. (Fabaceae) on the cell cycle of Allium cepa L. An Acad Bras Cienc. 2014;86:1147–50. http://dx.doi.org/10.1590/0001-3765201420130163
18. De Bona AP, Batitucci MCP, Andrade MA, Riva JAR, Perdigão TL. Phytochemical and mutagenic analysis of leaves and inflorescences of Erythrina mulungu (Mart. Ex Benth) through micronucleus test in rodents. Rev Bras Pl Med. 2012;14:344–51 (in Portuguese). http://dx.doi.org/10.1590/S1516-05722012000200014
19. de Queiroz FM, Matias KWO, Cunha MMF, Schwarz A. Evaluation of (anti)genotoxic activities of Phyllanthus niruri L. in rat bone marrow using the micronucleus test. Braz J Pharm Sci. 2013;49:135–48. http://dx.doi.org/10.1590/S1984-82502013000100015
20. Magalhães EA, Silva Júnior GJ, de Campos TA, Silva LP, Silva RMG. The evaluation of the genotoxic potency of the Pyrostegia venusta (Ker Gawl.) Miers, Bignoneaceae, crude extract on bone marrow of mice. Rev Bras Farmacogn. 2010; 20:65–9 (in Portuguese). http://dx.doi.org/10.1590/S0102-695X2010000100014
21. Miller LC, Tainter ML. Estimation of the ED_{50} and its error by means of logarithmic-probit graph paper. Exp Biol Med. 1944;57:261–4. http://dx.doi.org/10.3181/00379727-57-14776
22. Guerra M, de Souza MJ. How to observe the chromosomes: a guide to techniques in plant, animal and human cytogenetics. Ribeirão Preto, SP, Brasil: FUNPEC; 2002 (in Portuguese).
23. Marques RG, Morales MM. Petroianu A. Brazilian law for scientific use of animals. Acta Cir Bras. 2009;24:69–74. http://dx.doi.org/10.1590/S0102-86502009000100015
24. Procedures for the scientific use of animals. Subsection VII of § 1 of art. 225 of the Federal Constitution, Law no. 11. Brasilia, DF, Brazil; 2008. Avaliable from: http://planalto.gov.br/ccivil_03/_ato2007-2010/2008/lei/l11794.htm (in Portuguese).
25. Gomes KMS, de Oliveira MVGA, Carvalho FRS, Menezes CC, Peron AP. Cytotoxicity of food dyes Sunset Yellow (E--110), Bordeaux Red (E-123), and Tatrazine Yellow (E-102) on Allium cepa L. root meristematic cells. Food Sci Technol. 2013;33:218–23. http://dx.doi.org/10.1590/S0101-20612013005000012
26. Aissa AF, Bianchi MLP, Ribeiro JC, Hernandes LC, de Faria AF, Mercadante AZ, Antunes LMG. Comparative study of β-carotene and microencapsulated β-carotene: evaluation of their genotoxic and antigenotoxic effects. Food Chem Toxicol. 2012;50:1418–24. http://dx.doi.org/10.1016/j.fct.2012.02.030
27. Ribeiro JC, Antunes LMG, Aissa AF, Darin JDC, De Rosso VV, Mercadante AZ, Bianchi MLP. Evaluation of the genotoxic and antigenotoxic effects after acute and subacute treatments with açai pulp (Euterpe oleracea Mart.) on mice using the erythrocytes micronucleus test and the comet assay. Mutat Res-Gen Tox En. 2010;695:22–8. http://dx.doi.org/101016/j.mrgentox.2009.10.009
28. Demir E, Kocaoğlu S, Kaya B. Assessment of genotoxic effects of benzyl derivatives by comet assay. Food Chem Toxicol. 2010;48:1239–42. http://dx.doi.org/10.1016/j.fct.2010.02.016
29. Whittaker P, Clarke JJ, San RHC, Begley TH, Dunkel VC. Evaluation of the butter flavoring chemical diacetyl and a fluorochemical paper additive for mutagenicity and toxicity using the mammalian cell gene mutation assay in L5178Y mouse lymphoma cells. Food Chem Toxicol. 2008;46:2928–33. http://dx.doi.org/10.1016/j.fct.2008.06.001
30. Mpountoukas P, Pantazaki A, Kostareli E, Christodoulou P, Kareli D, Poliliou S, et al. Cytogenetic evaluation and DNA interaction studies of the food colorants amaranth, erythrosine and tartrazine. Food Chem Toxicol. 2010;48:2934–44. http://dx.doi.org/10.1016/j.fct.2010.07.030
31. Türkoğlu Ş. Genotoxicity of five food preservatives tested on root tips of Allium cepa L. Mutat Res-Gen Tox En. 2007;626: 4–14. http://dx.doi.org/10.1016/j.mrgentox.2006.07.006
32. Salinas RD. Food and nutrition: introduction to food science. Porto Alegre, RS, Brazil: Artmed; 2002 (in Portuguese).
33. Polônio MLT, Peres F. Food additive intake and health effects: public health challenges in Brazil. Cad Saúde Pública. 2009;25:1653–66 (in Portuguese). http://dx.doi.org/10.1590/S0102-311X2009000800002